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14. ABSTRACT

This project is intended to develop the tools and principles necessary to engineer subtilisin proteases which specifically target and deactivate biological warfare agent (BWA) toxins. We are engineering and evolving subtilisin proteases that specifically target and deactivate BoNT, SEB, ricin, and B. anthracis lethal factor (LF), representing four functionally distinct families of toxins. The centerpiece of our design effort is a phage-display selection method for creating tightly-regulated proteases of high specificity. In this system the protease, substrate sequence, and regulatory co-factor are co-evolved. The key accomplishments this past year were: 1) Design/evolution of a highly active enzyme that can cut P4 = I; 2) Design/evolution of an enzyme which can efficiently cut at P1 = Q; 3) First demonstration of the evolution of specificity for an ionic P4 amino acid (P4 = E); 4) Engineering protease chain reactions that can reliably measure concentrations in the 0.1 to 10 pM range.

15. SUBJECT TERMS

Enterotoxin, protease, directed evolution, subtilisin, protein engineering, phage-display, enzymology

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INTRODUCTION

This project is intended to develop the tools and principles necessary to engineer subtilisin proteases which specifically target and deactivate biological warfare agent (BWA) toxins. We are engineering and evolving subtilisin proteases that specifically target and deactivate BoNT, SEB, ricin, and B. anthracis lethal factor (LF), representing four functionally distinct families of toxins. The ability to facilely engineer enzymatic function will lead to enormously powerful, biologically-inspired materials. Serine proteases are among the most studied and best-understood enzymes and offer unique opportunities for progress. Serine proteases of the chymotrypsin and subtilisin families became early model systems for protein engineering because of well-characterized mechanisms, timely cloning of the genes, ease of expression and purification, and the availability of high-resolution atomic resolution structures. There are several excellent reviews of these early studies (*1-3*). Although the concept of evolving protease specificity might appear simple, the mechanistic knowledge of proteases required to engineer their specificity turns out to be very complex. Early protein engineering experiments provided many important insights and concepts, but also identified numerous challenges with which we still struggle. Our basic premise in this project is that we can do much better if existing knowledge is carefully applied.

To place our results in a larger context, we will briefly review some classical experiments on chymotrypsin-like and subtilisin-like proteases. These are well-studied proteases which are representative of large families. These proteases have been the starting point for many insightful engineering experiments in which both careful kinetic and structural analyses have been performed. We will attempt to define the current state of the art and identify the challenges which must be addressed to further advance the field. We emphasize the earliest studies in particular because they show that many of the obvious strategies for manipulating specificity were carried out in the 1980's soon after methods to introduce site-directed mutations became widely accessible.

Interpretation of kinetic data Below is a minimal realistic mechanism for peptide hydrolysis by a serine protease:

$$\mathbf{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathbf{ES} \xrightarrow{k_2} \mathbf{EA + P_1} \xrightarrow{k_3} \mathbf{EP_2} \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} \mathbf{E + P_2}$$

1 **2** **3** **4**

The reaction can be divided into four phases: 1) substrate binding; 2) acylation and release of the C-terminal peptide (P_1), 3) deacylation and 4) dissociation of the N-terminal peptide (P_2). Nucleophilic attack of the carbonyl carbon of the scissile amide bond is carried out by the active site serine. The other two amino acids forming the catalytic triad are histidine and aspartic acid which form a charge relay system. Serine proteases have evolved to manage the burial of charged groups during the catalytic cycle. In the enzyme-substrate complex, the catalytic aspartic acid forms a very strong H-bond to Nδ1 of histidine which polarizes the histidine and allows Nε2 to act as a proton shuttle during acylation and deacylation reactions.

Measuring specificity Typically steady state kinetic measurements are used to assess the specificity of a protease. Specificity is usually defined as the ratio of k_{cat}/K_M of an enzyme for one substrate relative to another. Determining k_{cat}/K_M values for two substrates allows quantitation of sequence preferences but does not reveal the kinetic and thermodynamic basis for the preference (*4*). To understand the mechanistic basis for specificity, transient state kinetic methods must be employed to determine microscopic rate constants. It is important to understand that K_M and k_{cat} are composite rate constants into which are folded multiple microscopic rate constants for the multi-step hydrolysis

reaction. It frequently is assumed for many enzymatic reactions that $k_{cat} \sim k_2$ and $K_M \sim K_S$. These relationships are accurate only if k_2 is small compared to k_{-1}, k_3 and k_4 however. As k_2 approaches k_{-1}, substrate binding can no longer be viewed as a rapid equilibrium which is kinetically uncoupled from acylation. This has important consequences for specificity. The k_{cat}/K_M value is the apparent second order rate constant for productive substrate binding. It is less than the true binding rate (k_1) by a factor of $k_2/(k_{-1} + k_2)$ (*4*). As k_{-1} slows to less than the acylation rate and the enzyme begins to reach a maximum determined by the rate of substrate binding, as the coefficient $k_2/(k_{-1} + k_2)$ approaches one. Thus coupling between substrate binding and acylation (the first chemical step) broadens specificity. Further, as product release becomes slower than acylation, it determines the k_{cat} of the reaction rather than the acylation rate.

Binding interactions Substrate-enzyme interactions are well characterized for both subtilisin and chymotrypsin-type proteases from high resolution x-ray structures of many protease-inhibitor complexes (*5-8*). At first glance, engineering protease specificity may seem to be a problem of engineering lock and key fit between the protease and the substrate sequence one desires to cut. We observe, however, sequence-specific cleavage is much more subtle, depending upon how side chain interactions influence not only ground state binding but also the positioning in the scissile bond relative to catalytic amino acids.

In subtilisin, most contacts are with the first five substrate amino acids on the acyl side of the scissile bond (denoted P1 through P5, numbering from the scissile bond toward the N-terminus of the substrate (*9*)) and the first amino acid on the leaving group side (denoted P1'). The backbone of the substrate inserts between strands 100-104 and 125-129 of subtilisin to become the central strand in an anti-parallel β–sheet arrangement involving ten main chain H-bonds (*10, 11*). Hence, a major component of substrate binding energy involves the peptide backbone. The side chain components of substrate binding result primarily from the P1 and P4 amino acids (*12-14*). Optimal substrates for subtilisin have large hydrophobic amino acids at the S1 and S4 sub-sites of the enzyme (*12, 13*).

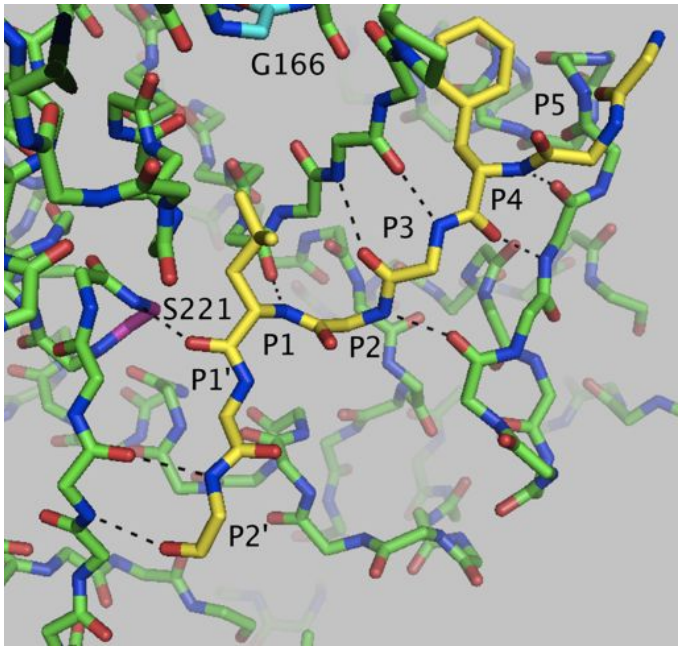


Figure 1. *Structure of a peptide substrate (yellow) spanning the subtilisin active site. Black dashed lines represent interactions represent main chain H-bonds between the peptide and the subtilisin binding cleft. The side chains of the P1 leucine and the P4 phenyalanine and shown. The position of the catalytic serine 221 is shown in pink as well as glycine 166 at the back of the S1 pocket. The depiction is based on 3BGO.pdb* (*15*).

In chymotrypsin, the P1 to P3 amino acids are also bound in an anti-parallel β–sheet arrangement with the strand 214-216 of the enzyme. Such anti-parallel beta strand interactions in the substrate backbone are typical of most proteases. The most intimate side chain interactions are with the P1 amino acid which is bound in a deep pocket comprising enzyme amino acids 189, 190, 216 and 226.

The pocket is deeper than either the S1 or S4 pockets of subtilisin with the P1 the side chain pointing directly into the enzyme. Trypsin, chymotrypsin and elastase form an extremely useful trio of proteases for understanding specificity and the potential to engineer new specificities. The three proteases have very different P1 specificities yet the different substrate specificities appear to be controlled by a small number of amino acids in the S1 binding pocket (Table 1). Four amino acids are conserved in each particular protease in this structural class.

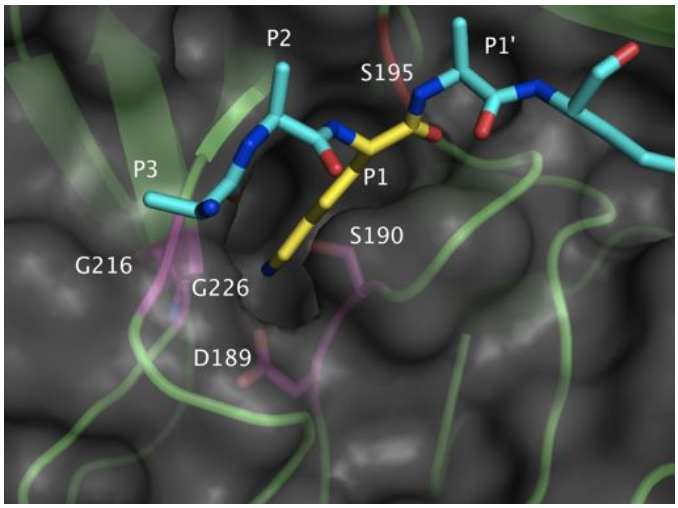


Figure 2. *Structure of a substrate (cyan) bound to trypsin. The side chains of key amino acids in the S1 pocket are labeled and shown in pink. The position of the catalytic serine 195 is shown in red. The depiction is based on 3FP8.pdb* (*16*).

Table 1

	S1 pocket	P1 specificity
Trypsin:	D189, S190, G216, G226	K/R
Chymotrypsin	S189, S190, G216, G226	large, hydrophobic
Elastase:	S189, G190, V216, T226	A

P1 specificity of chymotrysin-like proteases The structures and catalytic properties of trypsin, chymotypsin, and elastase seem to be text-book examples of how specificity can be inferred from structure and suggest seductively simple rationales for engineering specificity. The reality is much more complicated, as is extremely well reviewed in (*1, 2*) as well as Chapter 9 of (*17*). A series of well-thought-out experiments demonstrate that mutations in the S1 pocket alone cannot interconvert specificities. These experiments started with the S1 pocket and expanded mutations into more distal positions. These studies revealed four principles which frustrate the simple design strategies.

1. Both the S1 pocket and the surrounding area are fragile and easily deformed by mutation. In addition, mutations in the S1 site can change the backbone position of G216 which controls accurate positioning of scissile bond. This complicates design because one cannot assume a fixed backbone structure.

2. The natural design is subtle. For example, trypsin prefers P1 = R but cuts P1= K almost as well, even though the interaction of P1 = R makes a direct ion pair interaction with D189 while the interaction of P1 = K with D189 is via a water molecule. The rate for all other P1 amino acids is $\geq 10^5$ times slower.

3. Non-cognate P1 amino acids affect primarily k_2 rather than K_S. Thus P1 binding interactions can be translated into transition state stabilization rather than ground state stabilization in ways that are not apparent from high resolution structures.

4. Remote interactions contribute to catalysis in a variety of ways including solvent shielding, assisting in properly aligning the substrate to the catalytic triad, and by inducing distortions to the planar geometry of the scissile bond which stabilizes the transition state (*2*).

The S1 site of subtilisin The S1 pocket of subtilisin comprises amino acids, 127, 154, 156 and 166 and a water molecule that is hydrogen bonded to carbonyl oxygens of 126 and 152 and the main chain nitrogen of 169. Optimal substrates for wild type subtilisin have Y, F, L or M as the P1 amino acid (*12, 14*). Estell et al. performed a classic exploration of the interrelationship between the size and shape of the S1 pocket and the P1 specificity (*12*). In these experiments they varied the amino acid at position 166 of the enzyme and tested steady state kinetic parameters again substrate variations at the P1 position. The results follow a clear trend relating the volume and hydrophobicity of the S1 pocket and the size and hydrophobicity of the P1 amino acid. These studies were highly informative and satisfying in the simplicity of the trend identified. The observations were also consistent with the broad substrate specificity of subtilisins. Nevertheless, the type of linear change in specificity resulting from mutation makes engineering high specificity subtilisins somewhat daunting but for different reasons than those encountered with chymotrypsin.

Hydrophobic packing in the S1 site of subtilisin is in some ways reminiscent of the protein folding problem. In the folding analogy, sub-site variation is viewed as mutation. Changes in P1 generally result in significant but not catastrophic losses in transition state stability. Among hydrophobic P1 amino acids, the k_{cat}/K_M for P1 = Y is the best and P1 = A is 100-times less. k_{cat}/K_M values for the remaining hydrophobic amino acids span the range in between. In the same way, a mutation in the hydrophobic core of a protein may decrease stability but is frequently not catastrophic because of adjustments in neighboring amino acids.

To put the design problem into perspective, imagine designing a protein which is stably folded with one specific amino acid at a given position but unfolded with the other 19 amino acids at that position. This is obviously a much more challenging problem than just designing stabilizing or destabilizing mutations. This is basically what we would like to do in engineering protease specificity, however. Ideally one would like to engineer a sub-site so that only one amino acid supports catalysis. One way around this dilemma is to engineering disqualifying interactions at a sub-site – that is engineer interactions with non-cognate amino acids which are catastrophic. Steric clashes are one possible type of disqualifying interaction. In fact Van der Waals overlaps are the strongest non-covalent force associated with protein-protein interactions and create the possibility of decoding the binding of substrate amino acids which are too big to fit. Estell et al also examined the specificity of mutants with a large amino acid at 166 (*12*). The mutant I166 is an interesting example. First of all this mutation results in a preference for P1 = A or V. Also this mutation results in a large decrease in activity vs. P1 = F or Y, consistent with the idea of steric exclusion. The decrease in activity against intermediate P1 amino acids such as P1 = M, L or H relative to P1 = A is much smaller and indicates the ability of the P1 amino acid to adjust to the sub-site environment. This tendency of the S1 sub-site and the P1 amino acid to adjust to each other is also clearly documented with α–lytic protease (*18*).

Another type of disqualifying interaction involves ion pairs such as the P1 specificity for K or R observed in trypsin. The engineering challenge is that buried salt bridges are rare in nature and hard to engineer because the energy gained from the internal salt bridge must pay the cost of desolvation of the charged groups and also must compensate for lost interactions with counter-ions in solution. Wells et al. carried out studies which introduced charged amino acids in positions 156 and 166 of the S1 pocket (*19*). Natural subtilisins are very poor at cutting substrates with an acidic P1 amino acid. Subtilisin can be made about 400-fold better vs. P1 = E by mutating E156S and G166K. The activity vs. P1=M is about 100-fold higher than P1 = E, however. Introducing a negatively charged amino acid at 166 (G166E) actually decreases activity for P1=K, but since it also decreases the activity for P1= M, the net result four-fold preference for P1 = K over M. For much more on engineering subtilisin to cleave basic recognition sequences see (*20, 21*).

These experiments in subtilisin were carried out before structures were determined for the eukaryotic subtilisins Kex2 (yeast) and furin (human). These eukaryotic subtilisins are highly selective for arginine at the P1 position so it is interesting to compare the subtilisin engineered for a basic P1 amino acid with natural ones. The S1 pockets of furin and kex2 are similar to each other and much more exotic than the engineered subtilisin (*22, 23*). First the path of the main chain at the back of the S1 pocket has receded from its position in subtilisin. As a result the pocket is bigger and there is no amino acid equivalent to the key 166 amino acid in subtilisin. Second, and counter-intuitive to the binding of a basic P1 amino acid, a calcium ion is bound at the back of the pocket. The calcium is involved in a complex coordination network with three aspartic acids, a glutamic acid, three water molecules and the P1 Arginine (**Figure 3**). The prohormone-processing subtilisins also lack the structural equivalent of the 100-104 strand (*22, 23*). The acylation rates of Kex2 and furin are very fast with cognate substrates and dramatically fall with near-cognate sequences. Both substrate binding and transition state stabilization are dependent on the binding of calcium in the in S1 pocket (*24-28*). Co-factor dependent catalysis appears to tightly link substrate binding energy to transition state stabilization (*29*).

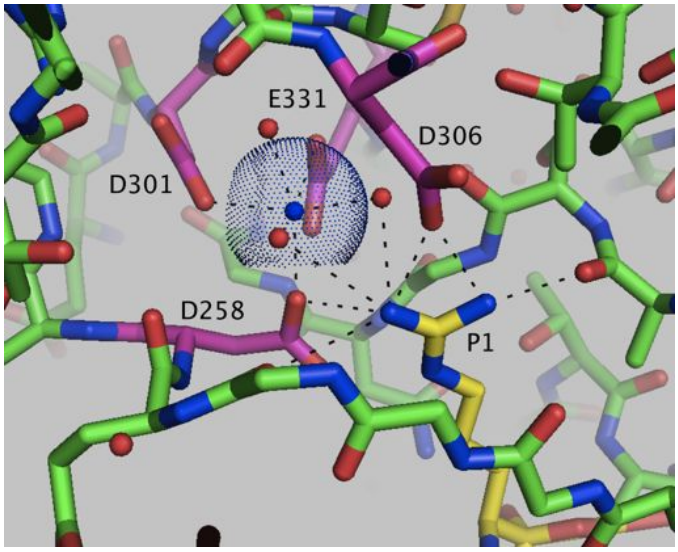


Figure 3. *Structure of a P1 arginine (yellow) bound to its subsite in furin. The side chains of acidic amino acids in the binding pocket are labeled and shown in pink. The bound calcium is shown in blue and water molecules coordinated to the calcium are shown in orange. Black dashed lines represent selected interactions under 3 Å. The depiction is based on 1P8J.pdb* (*23*).

A more recent protein engineering experiment involved the engineering ionic interactions at the S1 site to convert subtilisin into an enzyme which accepts phosphotyrosine at the P1 site by introducing the mutations E156R and P129G (*30*). Natural subtilisin is very poor against P1 = phosphotyrosine, as well as aspartic and glutamic acid. In the engineered enzyme the activity for phosphotyrosine increased by 500-fold relative to the wild type enzyme (k_{cat}/K_M). This is very good. Activity against tyrosine at P1 remains greater than 10^6 $M^{-1}s^{-1}$, however.

The S4 site of subtilisin The S4 site of subtilisin comprises amino acids at positions 104, 107, 126, 128, 130, 132 and 135. The natural preference of subtilisin for a P4 amino acid is as follows: F > L ~ V ~ I > A (*31-34*). A small P4 amino acid, such as alanine, points into the enzyme, but larger ones such as M, F, or Y, lie along a shallow indentation in the enzyme surface. The preference for F relative to A is about 3- fold. (*33, 35*). In subtilisin BPN', the S4 pocket has additional capacity, somewhat occluded behind the Tyr 104 residue. This additional capacity in S4 is the basis for the wild type's ability to turn-over substrates with Phe at P4 slightly faster than substrates with Ala at P4. Y104 is able to adjust its position to accommodate larger or smaller amino acids. Many other subtilisins have V104 rather than Y.

Very thorough studies of S4 mutations coupled with P4 substrate variations have been carried by several groups (*8, 13, 31, 32, 34, 36-40*). The analysis is an interesting complement to the analysis of S1 mutations because the S4 site is distal to the scissile bond. Most of the S4 mutations analyzed make the S4 site bigger. The greatest gains in specificity are from decreasing activity for P4 = A, relative to larger hydrophobic amino acids as the pocket is expanded. For example, Rheinnecker et al.

(*33*) have shown that expanding the S4 pocket can result in a significant increase in specificity by affecting both K_M and k_{cat}. For example, each of the single mutations Y104A and I107G result in a 200-fold preference for P4 = F vs. A. They suggested that the expanded S4 cavity causes structural changes which are propagated to the active site. Optimal active site geometry is restored by filling the cavity with a bulky P4 hydrophobe.

Gron et al. performed an S4 analysis which was conceptually similar, but used longer substrates in evaluating sub-site preferences (*14*). This analysis revealed the specificity at one sub-site can strongly depend on substrate amino acids at other sub-sites. For example, most early subtilisin analysis was performed with small substrates with P2 = proline. This minimizes non-productive substrate binding modes and simplifies analysis. However, P2 = is also a poor P2 amino acid which removes a main chain H-bond with G100. This weakens substrate binding, which in turn make catalysis more sensitive to substrate variations at other sites. Gron et al showed that a plateau in k_{cat}/K_M is reached as substrate interactions at other sub-sites are optimized. Subtilisin cleaves preferred substrates at a rate of ~1e7$M^{-1}s^{-1}$ (*13*).

Kinetic coupling and specificity A common assumption in enzyme engineering is that substrate binding is in rapid equilibrium and that the first chemical step (acylation for serine proteases) is rate limiting. These assumptions are often considered axiomatic for subtilisins, but in fact are not true for many substrate sequences (*41*). As substrate binding improves, these assumptions break down. This principle was illustrated using transient state kinetic experiments to analyze specificity in a Y104A subtilisin (which prefers substrates with phenylalanine or tyrosine at the P4 position) (*42*). While highly selective substrate binding was achieved in the Y104A mutant, several factors cause sequence specificity to fall far short of that observed with natural processing subtilisins. First, for substrate sequences which are nearly optimal, the acylation reaction becomes faster than substrate dissociation. As a result, discrimination among these substrates diminishes due to the coupling between substrate binding and the first chemical step (acylation). Secondly, although the engineered mutant has 24 fold higher substrate affinity for an optimal substrate (DFKAM) vs. a near-cognate sequence (DVRAF), the increase substrate binding energy is not translated into improved transition state stabilization of the acylation reaction. Finally, as interactions at subsites become stronger, the rate determining step in peptide hydrolysis changes from acylation to product release. Thus the release of the product becomes sluggish and leads to a low k_{cat} for the reaction. This also leads to strong product inhibition of substrate turn-over as the reaction progresses. These results illustrate that to create higher specificity proteases, the effect of mutations on the entire reaction pathway should be considered.

BODY

Engineering/evolution of the S4 site

A major focus this past year has been to evolve specificity toward sequences identified by USAMRIID in two of the target toxins. Using an exploratory protease provided by Potomac (pS189), USAMRIID unambiguously identified the following cut sites:

BoNT/B FFMQ-S (exposed loop)

SEB INSH-Q (exposed loop)

In the last year, we have been engineering/evolving specificity toward P4 = I (SEB cut site). Given the fact that our initial random library did not identify mutants with the ability to cleave P4 = I, we are re-designing the S4 site using computational modeling to identify a better starting place in mutational space. The original pT1001 mutant has an S4 site that is long but shallow. A shallow, solvent-accessible sub-site appears to promote P4 promiscuity. In a series of mutants, we close off part of the pocket to form a short, shallow pocket. This design was based on phage selections of mutants cleaving the sequence GRAL. Having identified a short, shallow pocket in selections, we then open up space in the interior of the S4 site. This space is excluded from solvent in a substrate complex, forming a deep, buried pocket for the P4 amino acid.

To change the size and shape of the deep S4 pocket, we created variations at amino acid 107. This decision made by analyzing 107 variations in previous phage selections. We have examined five variations: 107 = L, I, V, A, G. In this series we change the shape of the pocket (L vs. I) and then systematically enlarge the site by removing one methyl group at a time from the amino acid at 107.

We have made these changes in combination with three different anion sites. This allows us to observe specificity in a series of mutants in which the acylation step becomes faster. In this series I30, P125 is the slowest, L30, P125 is moderate, and I30, S125 is fastest.

	S4 site	**Anion site (slow)**	
	107	**30**	**125**
pT2037	L	I	P
pT2043	I	I	P
pT2044	V	I	P
pT2045	A	I	P
pT2046	G	I	P

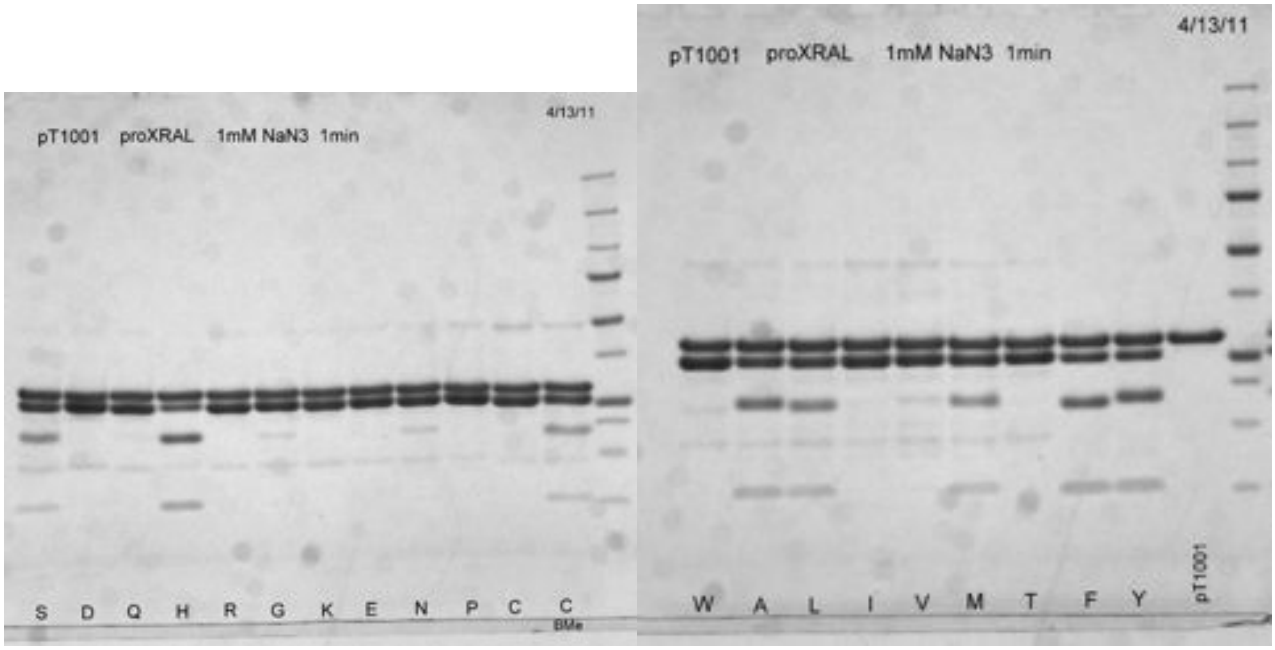
	S4 site	**Anion site (moderate)**	
	107	**30**	**125**
lethal	L	L	P
pT2047	I	L	P
not made	V	L	P
not made	A	L	P
pT2053	G	L	P

	S4 site	**Anion site (fast)**	
	107	**30**	**125**
lethal	L	I	S
pT2048	I	I	S
pT2049	V	I	S
pT2050	A	I	S
lethal	G	I	S

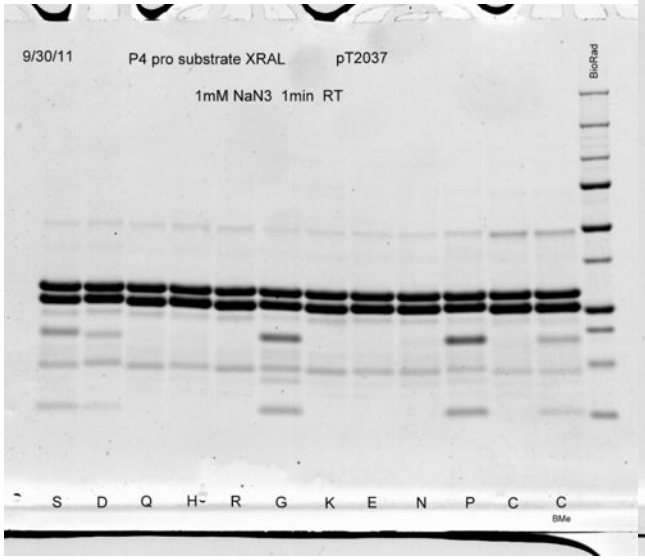
Quick summary:
I107 is most specific for A and G.
L107 Best vs. P4 = A, then P, G, T, S, V in that order.
V107 Best vs. P4 = A, then P and G, then M, S, T, V
A107 Best vs. P4 = A, then L, T, P, G and M, S, V
G107 -not yet determined but best candidate for cleaving Isoleucine.

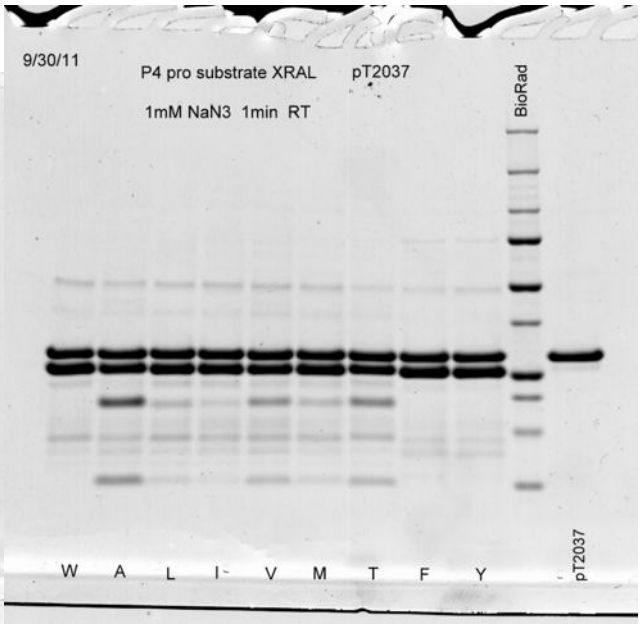
It may be noteworthy that we could not clone The P125S version of either L107 of G107.

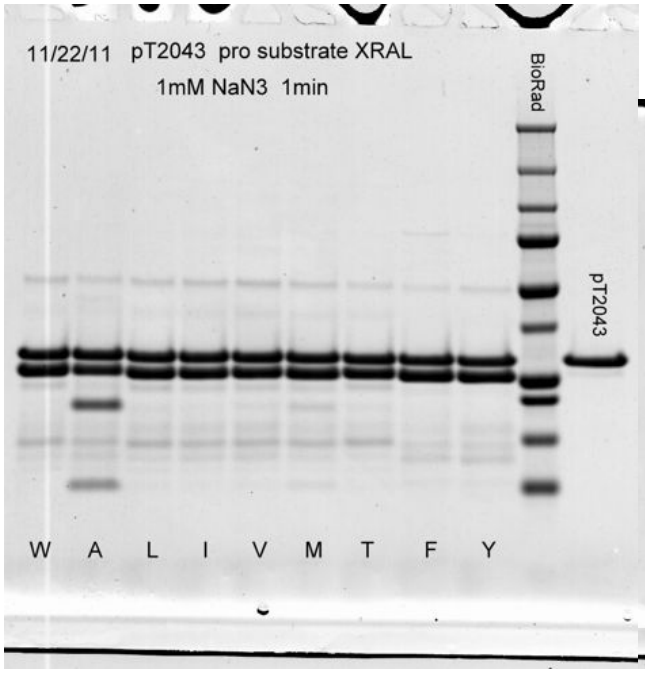
Original parent: Pro-substrate

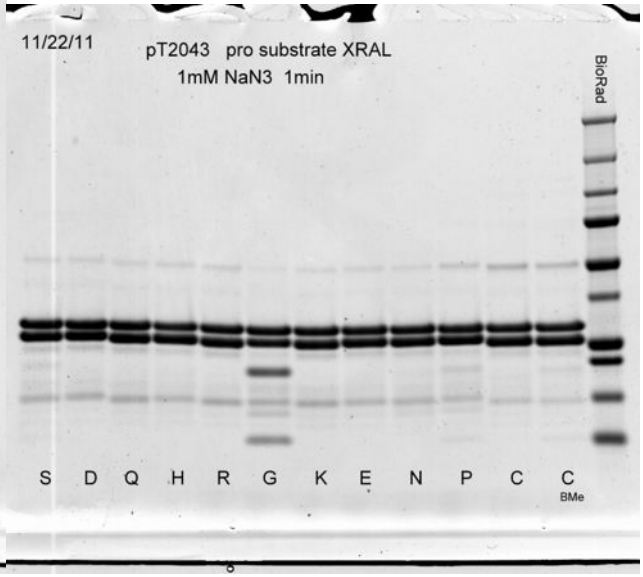


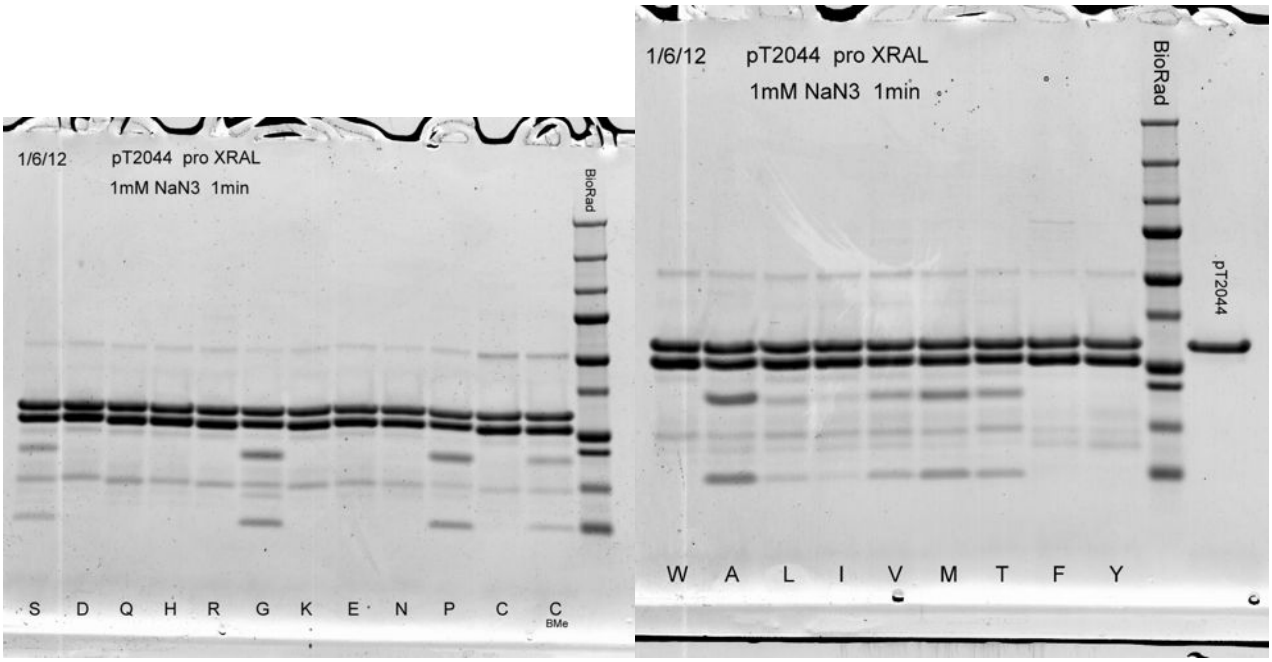
107 variations (slow anion site) pro-substrates







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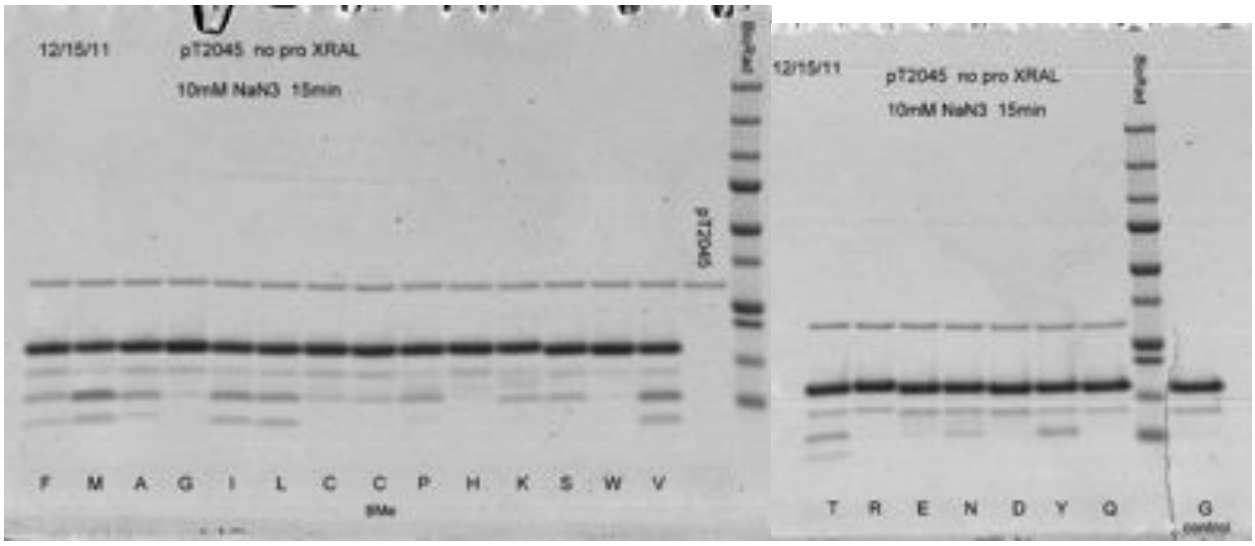
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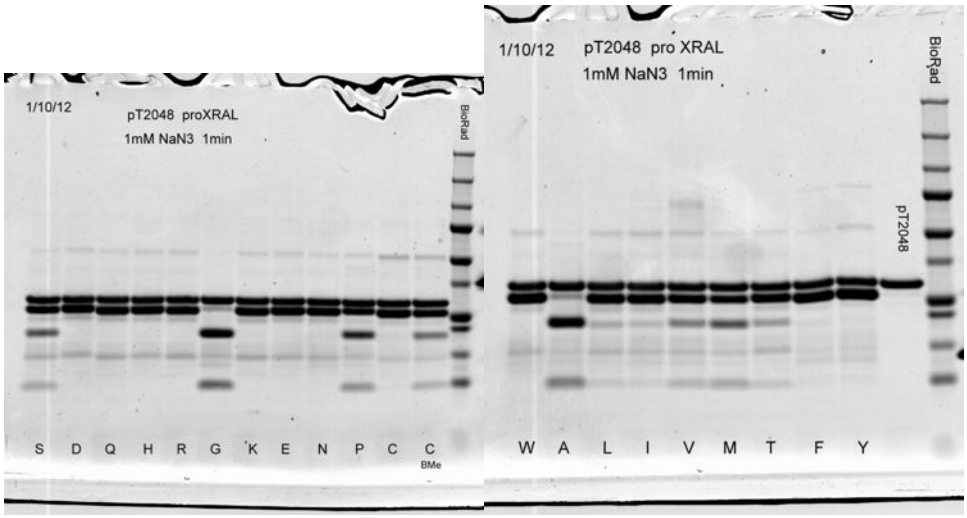
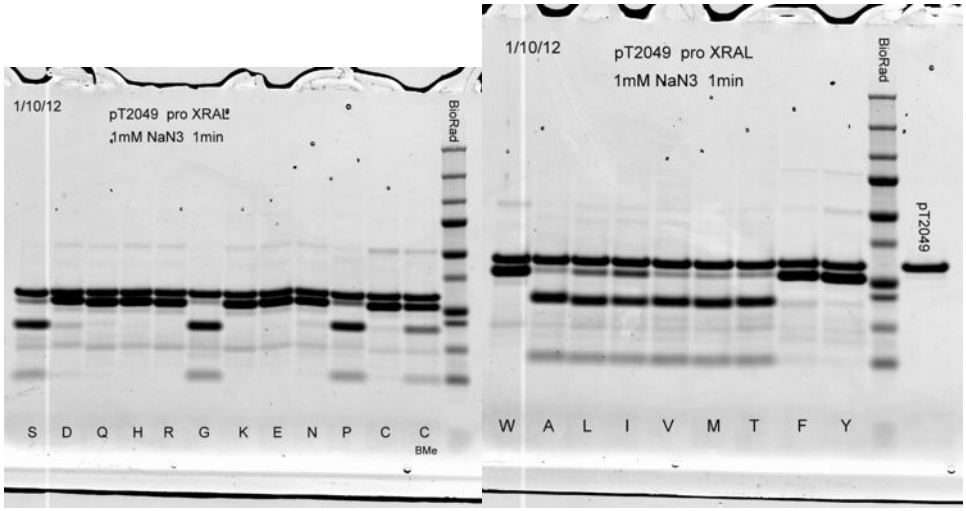
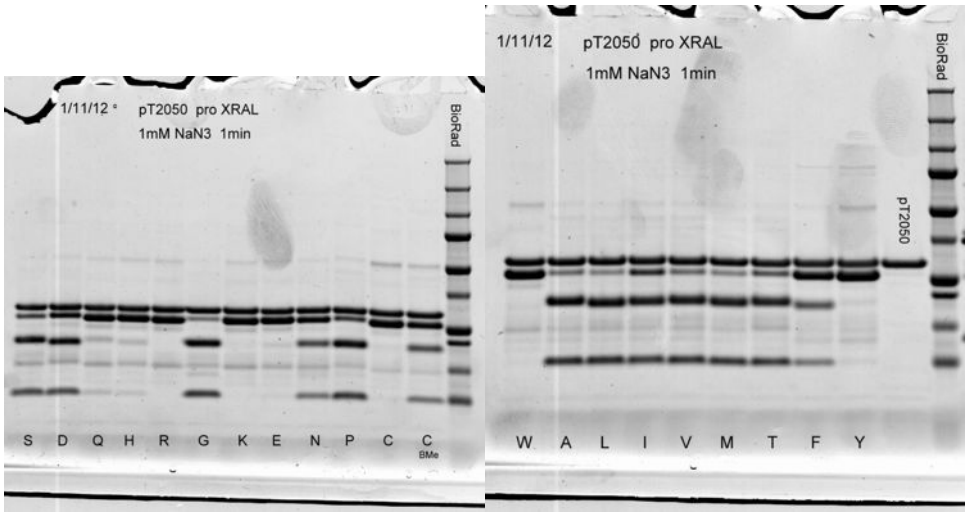
T R E N D Y Q G

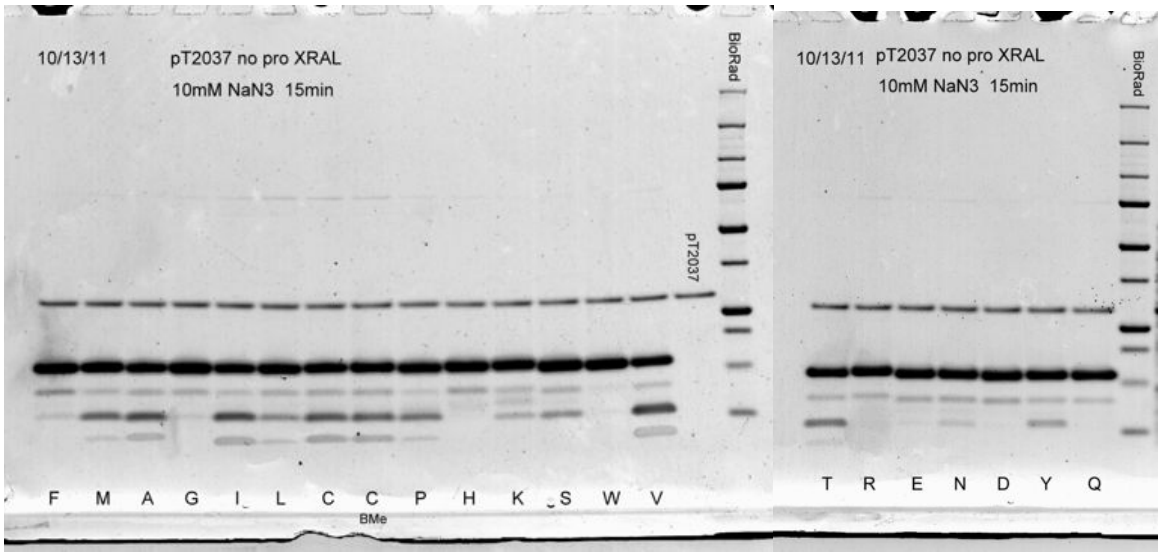
107 variations (fast anion site) Pro substrates

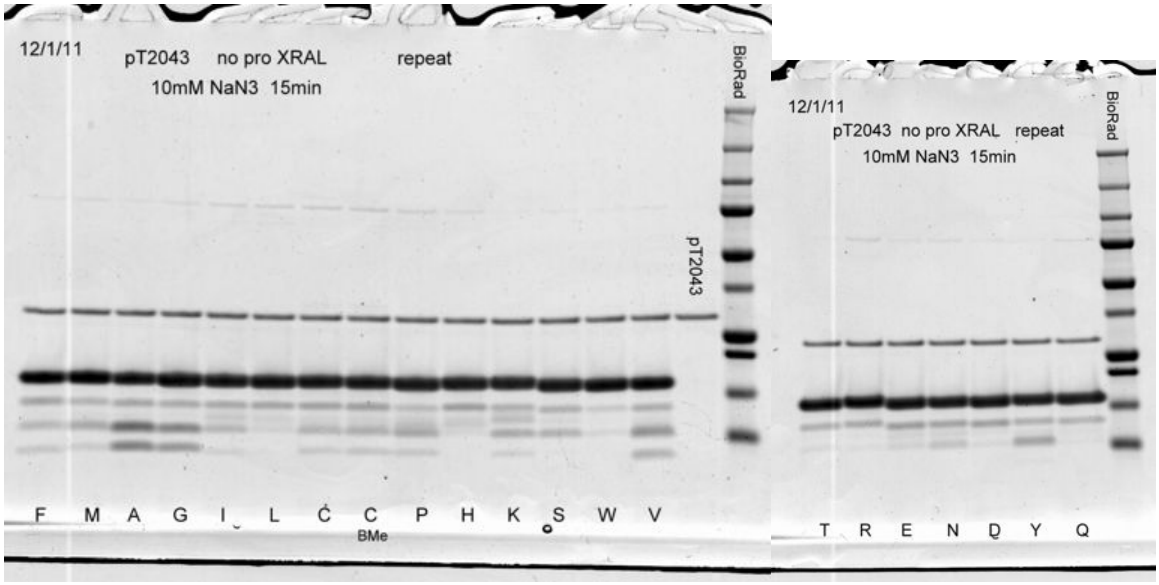




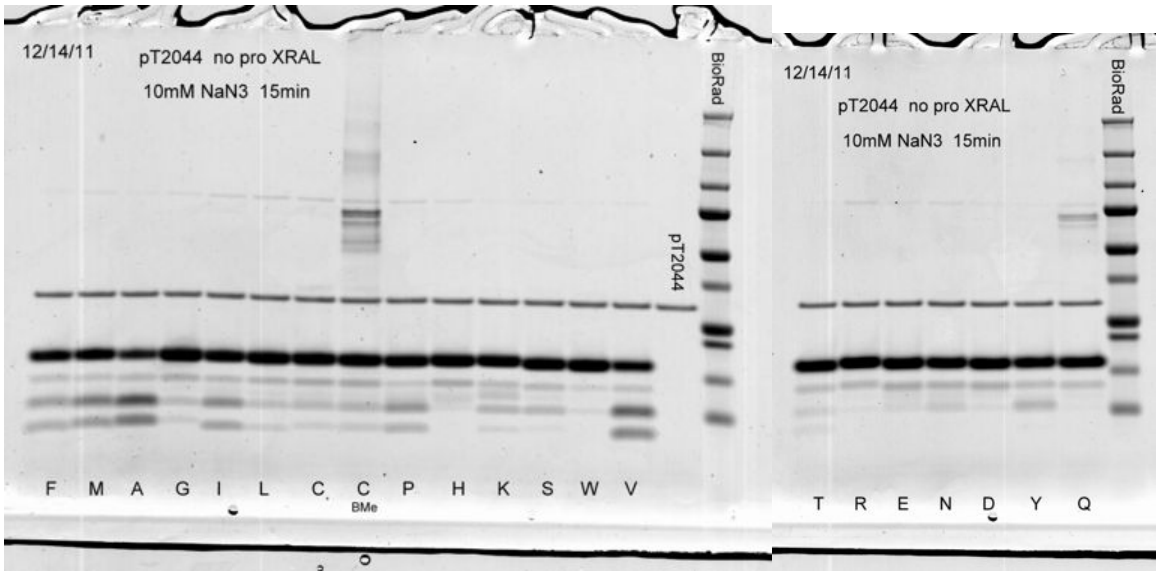


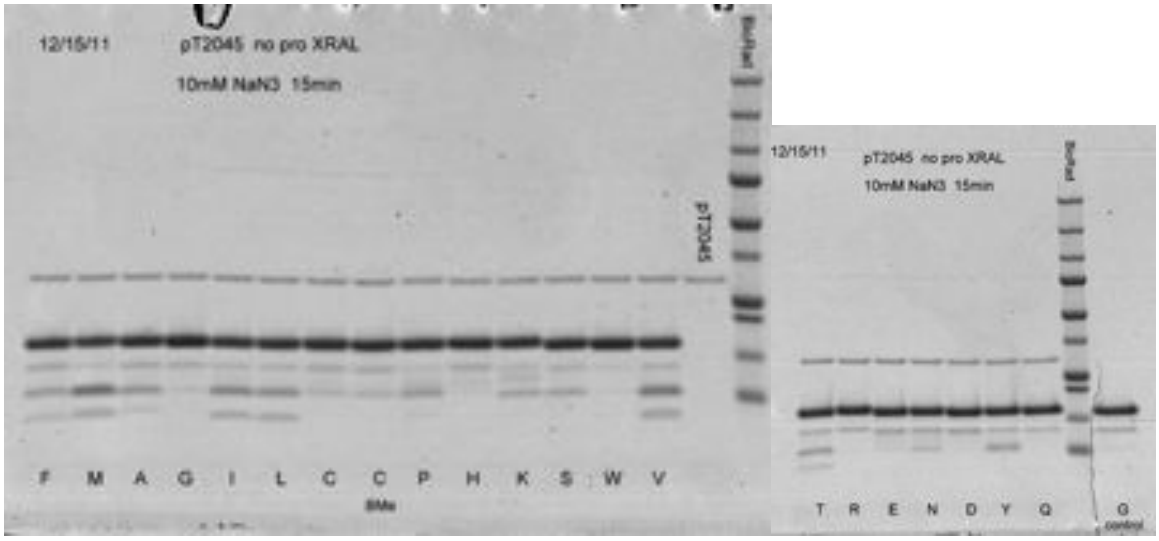
107 variations (slow anion site) <u>No-Pro</u> substrates



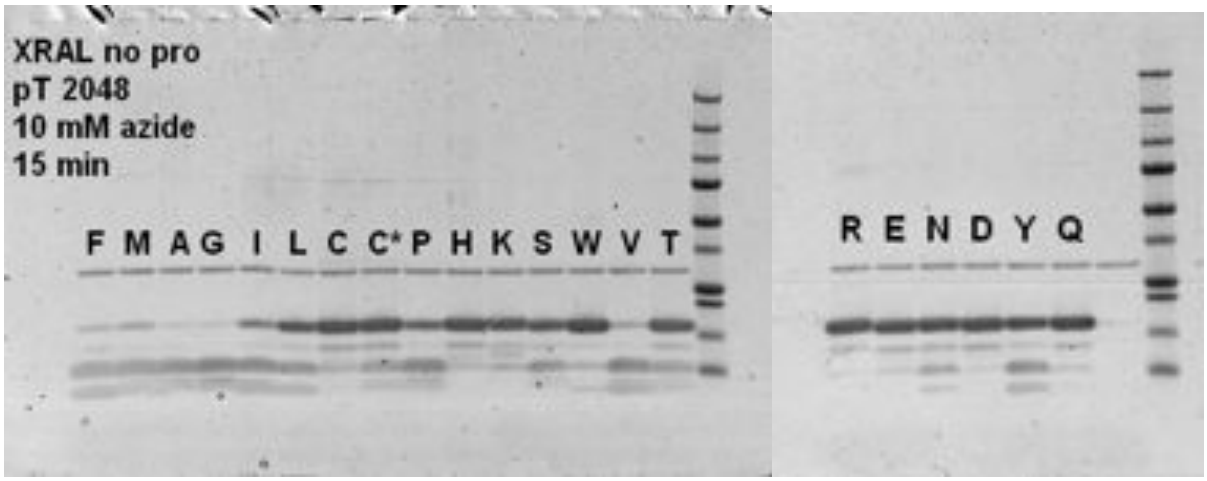


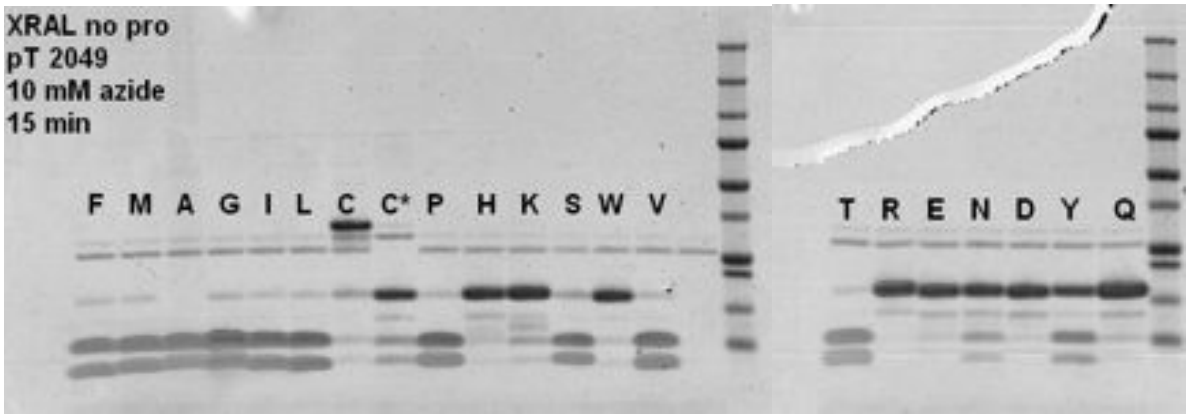
107 variations (slow anion site) <u>No-Pro</u> substrates

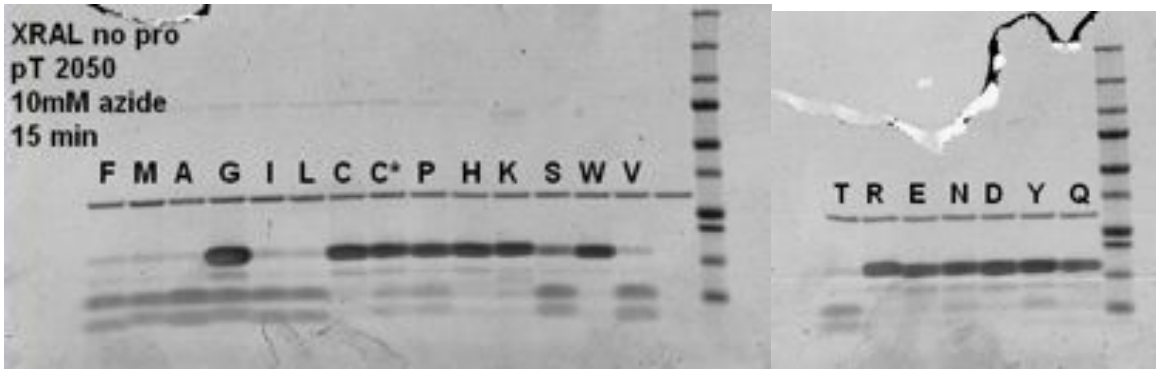




107 variations (fast anion site) <u>No-Pro</u> substrates







Additional iterations of S4 site engineering.

In the effort to reconfigure the long, shallow S4 binding pocket into a deep, buried pocket for hydrophobic P4 amino acids, we note two general trends that are potentially useful. 1) Many different mutations at the sites 104, 107, 132 and 135 can be introduced without compromising high activity for certain P4 amino acids. These sites constitute a variable environment, with the effect of mutations largely isolated to effects on interactions with the P4 side chain. 2) Most mutations at some sites (e.g. 126, 128) decrease activity against all substrates. These trends were considered in assembling the next series of S4 variants. As described above, we changed the size and shape of the deep S4 pocket by constructing and analyzing 107 variations (107 = L, I, V, A, G). We have also made with variations at 128 (128 = G, S, I) and in combination with variations at 104 (104= A, V, Y).

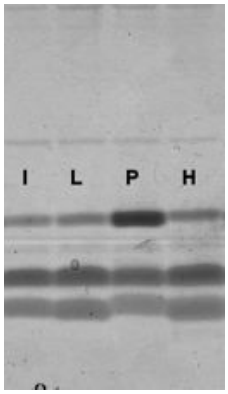
Our analysis has focused on 107 variations with the S128 with Y104 combination. In the presence of G128, Y104 can rotate away from the S4 pocket to allow adjustments for P4 amino acids of different size. Either S128 or I128 appear to prevent this adjustment in Y104 conformation. I128 was observed to generally reduce activity against all P4 amino acids. S128 was able to restrict Y104 conformation but preserves high activity against some P4 amino acids, thereby increasing specificity.

Table 2

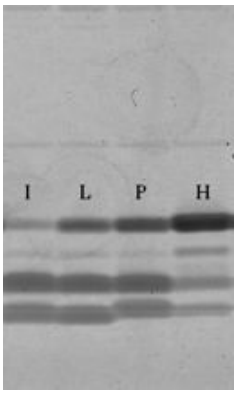
	104	107	128
pT2031	A	I	S
pT2057	Y	A	S
pT2058	Y	G	S
pT2059	Y	I	S

** 104V and 104L mutations were also attempted in this background but were found to be lethal to the E. coli host. Since they have potentially useful specificities, we are working on new vectors to allow their expression either in E. coli or B. subtilis.*

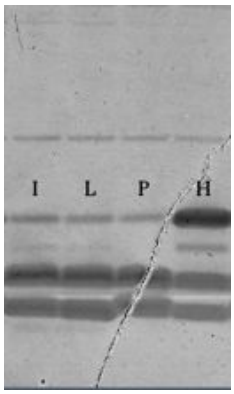
Kinetic analysis of the mutants defined in Table 2 is complete against the sequence P4 = X, P3 = R, P2 = A, P1 = L (where X = all twenty amino acids) in substrates with the full prodomain and without the prodomain. The preferred P4 amino acid for pT2032 is phenylalanine. The preferred P4 amino acids for pT2057-2059 are aliphatic amino acids. Below we highlight four amino acids with rough size complementarity.



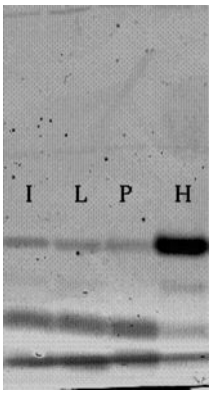
pT2031



pT2057



pT2058



pt2059

The shallow open site of pT2031 is able to bind some polar amino acids reasonably well. In contrast, pT2057-2059 cut polar amino acids very poorly. This is illustrated by their relative inability to cut P4 = H. HIstidine is an amino acid with aromatic character but also hydrogen bonding potential from the two

ring nitrogens. Because the P4 amino acid is completely excluded from bulk solvent in pT2057-2059, the unsatisfied hydrogen bonds result in diminished substrate binding.

The differential activity against P4 = P in this series is also noteworthy. Proline is a small hydrophobic amino acid that has no main chain amide proton because of the proline ring. The proline ring therefore eliminates a hydrogen bond between subtilisin residue 102 and the substrate backbone at P4. In pT2058-2059, improved side chain interactions compensates for the lost hydrogen bond. More kinetic analysis is needed to parse out finer details of activity among the aliphatic P4 amino acids.

Key finding: For the first time we have a highly active enzyme that can cut P4 = I with reasonable specificity (pT2057).

Engineering/evolution of the S4 site for P4=E

Using the example of the S1 site of furin as inspiration, we have design the S4 pocket of subtilisin to use a cation co-factor in order to create a specificity for a S1 glutamic acid. We had previously failed in several attempts to engineer and evolve ionic interactions at the S4 site. We introduced I107D and L135S mutations in pT2031 to create pT2067. pT2067 is highly specific for P4 = E. We believe that a cation is required to coordinate the binding of the acids P4 =E with D at 107. Analysis is underway to characterize the effect of diffent cations on catalysis and to further refine the design.

Key finding: For the first time we have an enzyme that can cut any charged P4 amino acid.

Engineering/evolution of the S1 site.

The process for re-engineering the S1 binding pocket is proceeding generally as described for the S4 pocket. We have introduced variations at amino acid 152 (at the base of the S1 pocket) and at 166 (at the top of the S1 pocket). Variations at 152 include A, G, and S. Variations at 166 include G, S, T, and D. This analysis is ongoing but we note that the mutant with S152 (pT1032) significantly increases activity against P1 = Q in the prodomain FRAX substrate series. This is significant in our goal to increase activity vs. the BoNT/B target sequence (FFMQ-S).

Key finding: We have an enzyme which can efficient cut at P1 = Q

Engineering cooperative binding interactions at S1 and S4.

A near-term goal is to create second generation random libraries of S1 and S4 binding pockets for phage display. Informed library design is critical for the success of this stage. Based on analysis of first generation phage selections and subsequent re-engineering by structure-based design, we believe that creating cooperativity between binding at S1 and S4 site has the potential to generate the highest specificity enzymes. The binding of a substrate to subtilisin appears to be a function of both the size and chemical complementarity of the side chain with a specific sub-site, as well as the global stability of the enzyme itself. The global enzyme stability comes into play because the beta strands comprising the peptide binding region can become distorted when destabilizing mutations are introduced even in distal regions of subtilsin. When a substrate binds, the beta strands reorganize into the canonical conformation. This reorganization is paid with substrate binding energy, weakening substrate binding. While this phenomenon complicates the interpretation of kinetic data, it can also potentially be exploited if substrate insertion and enzyme reorganization can be coupled in such a way as to cause cooperative binding interactions at sub-sites S1 and S4.

The S1 pocket, the S4 pocket and the anion site are all interconnected such that binding at one site can influence interactions at the other two. To promote this linkage we have mutated P168G. Proline at 168 is highly conserved in subtilisins and is in the rare cis conformation. By mutating this amino acid to

glycine, we create space at the apex of the loop that forms the backs of the S1 and S4 sites and we also destabilize the enzyme by replacing the rigid proline with the highly flexible glycine. This mutation was introduced into the backgrounds of S189 and S190 subtilisins. In these backgrounds, the mutation generally weakens substrate binding but has only a modest effect of specificity overall. A secondary effect is that the P168G mutation results in an amide proton deep in the S4 pocket, creating the potential for engineered polar interactions.

Engineering protease-base machines for detection and destruction of protein toxins

Proteases when bound to specific inhibitors acquire a unique potential to self-activate, self-amplify, and propagate signals. We have used engineered subtilisins and prodomain inhibitor variants to create synthetic protease chain reactions. In the engineering process, we create a series of components which can be combined to create programmable activation cascades. The basic components are formed from high-specificity, regulated subtilisins complexed with high affinity, but cleavable prodomain inhibitors. The protease is inactive when bound to the inhibitor but, once freed, is capable of cleaving the inhibitor and releasing additional free protease. A few molecules of free protease can initiate the chain reaction and will eventually cause the release of all protease from the inhibitory complex. Components were engineered in six steps: 1) Engineering/evolving a subtilisin tightly regulated by specific anions; 2) Engineering a subtilisin highly specific for a cognate sequence; 3) Engineering tight inhibition by the prodomain; 4) Engineering inhibitor release via an internal cleavage site in the prodomain that corresponds to the specificity of the engineered subtilisin; 5) Engineering subtilisins with incongruent specificities; 6) Engineering initiator subtilisins that are not inhibited by the prodomain.

Proteases with different specificities and tighter anion regulation can be assembled with prodomain inhibitors matched to a specific protease to improve sensitivity and accuracy of detection assays.

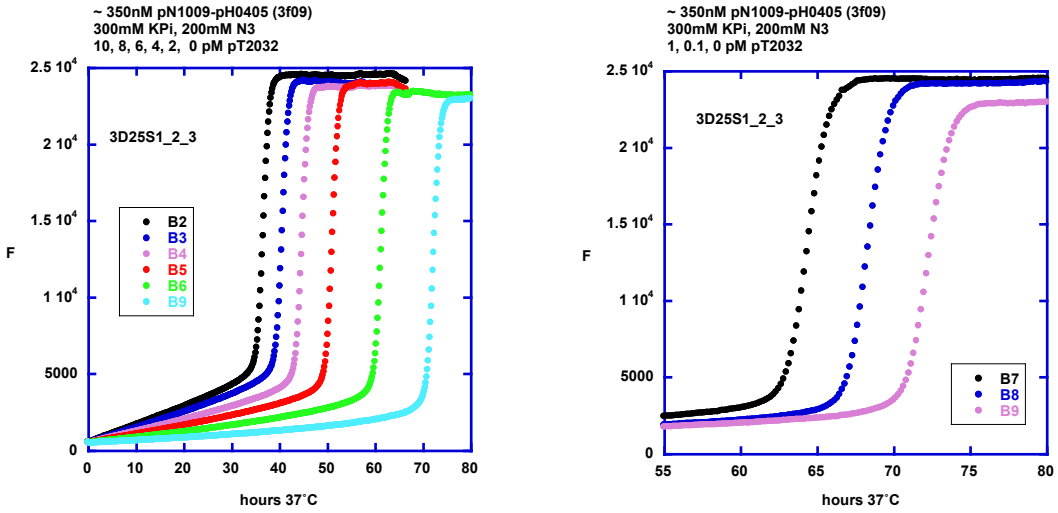


Figure 4

Key finding: We can reliably measure concentrations in the 0.1 to 10 pM range. This can now be done without any ramping step prior to the addition of all components.

Protease chain reactions can be used to measure the specificity of a protease

Previous kinetic analysis has been performed against substrates in which the target sequence with P4 variations occurs in an extended conformation. In the analysis described here, P4 variations are examined with the target sequence occurring in a structured loop (amino acids 18-21) connecting b-strand 1 and helix 1 of the prodomain (Figure 5, red loop).

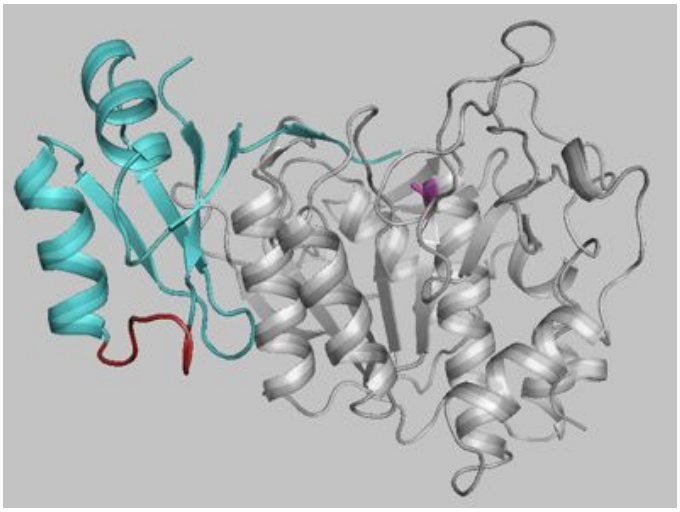


Figure 5 We constructed, expressed, purified nine prodomain loop variants with the P4 = F, H, I, M, L, A, V, P, or Y. The four proteases in Table 1 were mixed with each of the nine prodomain loop variants resulting in 36 complexes. Azide (20mM final) and fluorogenic substrate were added to each complex and the kinetics of self activation were determined. A representative reaction is shown in Figure 6.

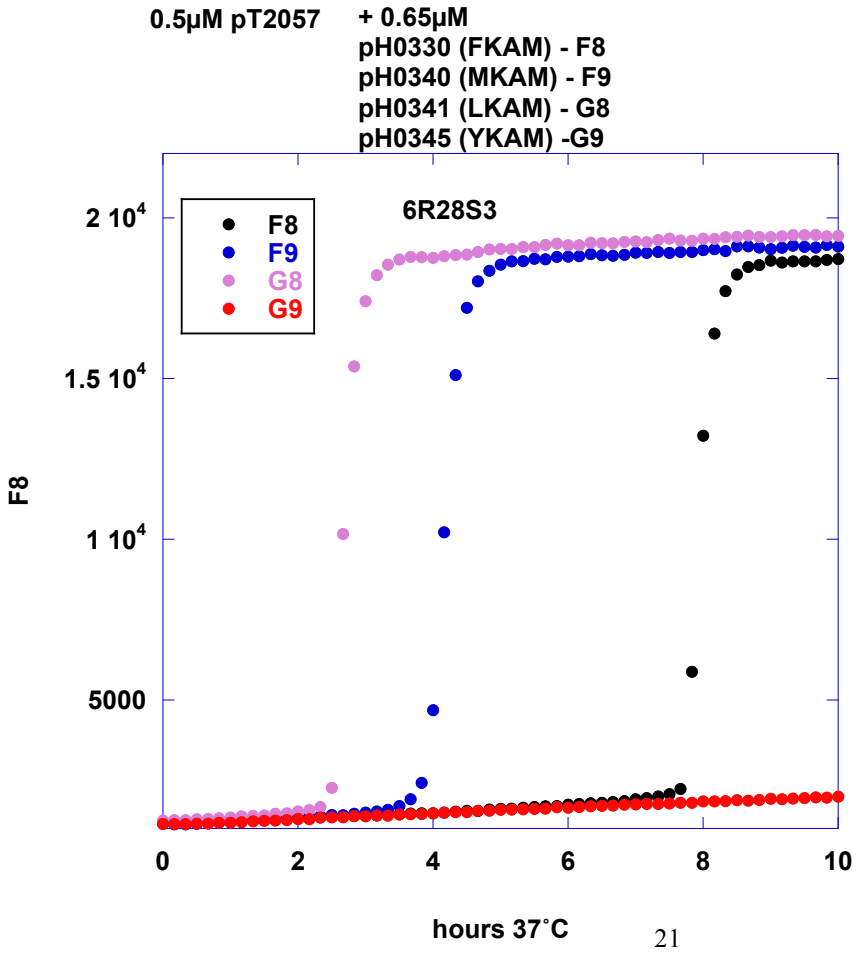


Figure 6 At time zero, complexes (0.5µM pT2057 and 0.65 µM prodomain) are mixed with azide (20mM final) and the fluorogenenic substrate sDFKAM-AMC (1µM final). The reaction is followed by fluorescence.

The key parameters determining the kinetics of self-activation are the inhibition constant of the prodomain and the rate of cleavage of the loop sequence by the subtilisin variant. These values were determined for each prodomain and protease combination using Kintek Global Explorer. These values are presented in Table 3.

Key findings: 1) The kinetics of loop cleavage are not the same as kinetics observed with substrates in an extended conformation. The loop cleavage assay should be more predictive of cleavage of exposed epitopes in proteins toxins. 2) We have identified proteases with orthogonal specificities (e.g. pT2031 cuts P4 = Y but not L and M), pT2057 cuts P4 = L and M but not Y). These orthogonal activities are of considerable use in constructing protease chain reaction cascades and will lead to greater sensitivity in detection assays.

Table 3

	Loop	Tail	Sbt	Ki (nM)	Time (hrs)	ΔF/10 min.	rate	Exp.	Well
1μM			0.5μM						
pH0330	FKAM	FRAL	pT2031	0.065	16.2	10000	7.8e4	6f15s2-3	B9
pH0338	HKAM	FRAL	pT2031	.089	No cascade			6t26s1	C2
pH0339	IKAM	FRAL	pT2031	0.077	No cascade			6f22s1	B8
pH0340	MKAM	FRAL	pT2031	0.075	No cascade			6f22s1	C8
pH0341	LKAM	FRAL	pT2031	0.087	No cascade	-----	----	6r14s1-2	H7
pH0342	AKAM	FRAL	pT2031	0.095	No cascade			6t26s1	C3
pH0343	VKAM	FRAL	pT2031	pending					
pH0344	PKAM	FRAL	pT2031	0.093	No cascade			6t26s1	C4
pH0345	YKAM	FRAL	pT2031	0.08	26.5	3000	3.9e4	6r28s1-2	F2
pH0330	FKAM	FRAL	pT2057	0.12	22.6	8222	3.1e4	6f15s2-3	B10
pH0338	HKAM	FRAL	pT2057	0.11	No cascade			6t26s1	C5
pH0339	IKAM	FRAL	pT2057	0.12	41.8	5200	1.71e4	6f22s1	B9
pH0340	MKAM	FRAL	pT2057	0.13	16.55	8422	3.9e4	6f22s1	C9
pH0341	LKAM	FRAL	pT2057	0.19	11.87	8467	3.75e4	6r14s1-2	H8
pH0342	AKAM	FRAL	pT2057	0.12	No cascade			6t26s1	C6
pH0343	VKAM	FRAL	pT2057	0.11	No cascade			6w20s1	A9
pH0344	PKAM	FRAL	pT2057	0.13	No cascade			6t26s1	C7
pH0345	YKAM	FRAL	pT2057	0.11	No cascade			6r28s1-2	F3
pH0330	FKAM	FRAL	pT2058	fast	2.08	10178	3.2e4	6f15s2-3	B11
pH0338	HKAM	FRAL	pT2058	0.85	No cascade			6t26s1	D2
pH0339	IKAM	FRAL	pT2058	1.27	6.67	4017	1.1e4	6f22s1	B10
pH0340	MKAM	FRAL	pT2058	fast	2.15	8694	3.4e4	6f22s1	C10
pH0341	LKAM	FRAL	pT2058	1.4	5.38	5833	1.2e4	6r14s1-2	H9
pH0342	AKAM	FRAL	pT2058	1.03	9.03	2117	1e4	6t26s1	D3
pH0343	VKAM	FRAL	pT2058	pending					
pH0344	PKAM	FRAL	pT2058	1.14	25.6	420	2.6e3	6t26s1	D4
pH0345	YKAM	FRAL	pT2058	0.81	13.4	1333	8.4e3	6r28s1-2	F4
pH0330	FKAM	FRAL	pT2059	fast	1.74	13200	5.4e4	6f15s2-3	B12
pH0338	HKAM	FRAL	pT2059	0.66	No cascade			6t26s1	D5
pH0339	IKAM	FRAL	pT2059	1.10	4.80	4883	1.7e4	6f22s1	B11
pH0340	MKAM	FRAL	pT2059	fast	1.45	10,367	5.7e4	6f22s1	C11
pH0341	LKAM	FRAL	pT2059	0.93	3.7	5522	2.6e4	6r14s1-2	H10
pH0342	AKAM	FRAL	pT2059	0.88	6.33	2483	1.6e4	6t26s1	D6
pH0343	VKAM	FRAL	pT2059	0.72	6.83	4333	1.8e4	6w20s1	A11
pH0344	PKAM	FRAL	pT2059	0.84	20.3	300	5.3e3	6t26s1	D7
pH0345	YKAM	FRAL	pT2059	0.65	9.8	1375	1.38e4	6r28s1-2	F5
0.65μM			0.5μM						
pH0330	FKAM	FRAL	pT2031	.044	4.9	9133	4e4	6r28s2	F6
pH0340	MKAM	FRAL	pT2031	0.049	No cascade			6r28s2	F7
pH0345	YKAM	FRAL	pT2031	0.046	11.2	4900	1.9e4	6r28s2	G2
0.65μM			0.5μM						
pH0330	FKAM	FRAL	pT2057	0.075	7.93	7338	1.7e4	6r28s3	F8
pH0340	MKAM	FRAL	pT2057	0.075	4.17	5517	3.1e5	6r28s3	F9
pH0341	LKAM	FRAL	pT2057	0.075	2.67	7890	4.5e4	6r28s3	G8

Protease chain reactions may be used to detect protease toxins

We have created and purified a series of prodomain mutants in which the recognition sequence of other sequence specific proteases is incorporated into the loop sequence of the prodomain (Figure 5). These are summarize in the Table.

	Loop sequence	specificity
pH0329	KQTFKAM-SA	pT2031
pH0334	KQTESAR-SA	trypsin
pH0335	KQTESAF-SA	chymotrypsin
pH0336	KKKKVYP-YP	Lethal Factor

The goal is to create a ProCR reaction that will detect and quantitate small levels on Anthrax Lethal Factor. Trypsin and chymotrypsin recognition loops are included as control reactions to aid in characterization.

Summary of progress on Statement of Work:

Task 1: Chose cognate sequences from target toxins

1.1 The awardees shall review existing BoNT, SEB, ricin, and LF protein structures for amino acid sequences that present likely targets for RSUB. (Y1Q1)

Completed

Task 2: Evolve anion-regulated protease specificity

2.1 The awardees shall create a GA-COGNATE-GB phage capture protein for Task 1-identified target sites on each of the four toxins. (Y1Q3)

Completed

Also created GA-COGNATE-GB phage capture proteins with individual sub-site variations:

P2 = all twenty *complete*

P4 = all twenty *complete*

P1 + all twenty *complete*

2.2 The awardees shall create a phage library for each of the RSUB candidates in which the P1' and P2 anion-binding regions have been randomized. (Y1Q4)

Completed

Three anion libraries created and screened:

Library 1: sites 32 33 62 68 125

Library 2: sites 33 62 96 123 125 126

Library 3: sites 123 124 125 126 222 224 225

2.3 The awardees shall use phage display to identify library members which exhibit optimized anion-triggered GA-GB cleavage of selected toxin target sites. (Y2Q1)

Completed

Anion libraries screened:

Library 1 screened vs.	pH0101	consensus sequence patterns obtained
Library 1 screened vs.	pH0106	no consensus pattern obtained
Library 1 screened vs.	P2 = X	no consensus pattern obtained
Library 2 screened vs.	pH0101	consensus sequence patterns obtained

2.4 Starting from the four anion-optimized proteases evolved in 1.2.3, the awardees shall create phage libraries in which the P1 and P4 protease sites have been randomized. (Y2Q2)

Completed

Three P4 libraries created:

Library 1:	sites	104	107	124	126	128	
Library 2:	sites	104	107	128	130	132	135
Library 3 (optimized phagemid):	sites	104	107	128	130	132	135

2.5 The awardees shall use phage display to select library members which exhibit the greatest specificity for each of the GA-GB capture proteins.(Y2Q3)

Completed

P4 libraries screened:

Library 1 vs. P4 = A	consensus sequence patterns obtained
Library 1 vs. P4 = F	consensus sequence patterns obtained
Library 1 vs. P4 = I	mostly deletions mutants obtained: phagemid vector system optimized to control fusion protein expression

Library 3 (optimized phagemid):	sites	104	107	128	130	132	135
Library 1 vs. P4 = G	consensus sequence patterns obtained						
Library 1 vs. P4 = Q	consensus sequence patterns obtained						

2.6 Starting from the four anion-optimized proteases evolved in 1.2.5, the awardees shall create and screen phage libraries in which the P3 and P5 protease sites have been randomized. (Y3Q1)

Not started

Task 3: Characterize catalytic properties of engineered proteases.

In progress

3.1 The awardees shall use subtilisin-Alexafluor 350 conjugates to measure protease kinetics with substrates containing each of the cognate toxin sequences. (Y3Q2)

Kinetics analysis underway with selected mutants from anion 1 library-pH0101 selection.

Promising mutants given to USAMRIID for testing with toxins.

Protease chain reaction assay developed to assay activity and specificity of sequences in a structured environment.

KEY RESEARCH ACCOMPLISHMENTS

1. Design/evolution of a highly active enzyme that can cut P4 = I (pT2057).
2. Design/evolution of an enzyme which can efficiently cut at P1 = Q (pT1032)
3. First demonstration of the evolution of specificity for an ionic P4 amino acid (P4 = E, pT2067);
4. Engineering protease chain reactions that can reliably measure concentrations in the 0.1 to 10 pM range.

REPORTABLE OUTCOMES

Bryan, P. N. (2012) *Engineering Protease Specificity*, in The Protein Engineering Handbook Vol. III, Lutz and Bornscheuer, eds., Wiley Press, Weinheim. (pp 243-278).

CONCLUSIONS

This projecting is developing tailor-made decontaminating and/or therapeutic agents with minimal adverse effects on humans or the environment. The success of the project can be judged on two levels. First, do our results have practical benefit for degrading protein toxins? Second, will our results make future enzyme engineering efforts easier? We have made significant progress toward both the primary goal as well as the second, broader goal of translating results and insights into general knowledge that will enable the creation to other custom enzymes. We are developing three broadly applicable concepts.

1. Engineering co-factor-dependent activity increases enzyme specificity. This principle is general to any multi-step enzymatic process, although often not considered by protein engineers. We have discussed this principle in detail in the first year's annual report.

2. Switchable enzymatic activity leads to selection methods that optimize transition state binding rather than substrate or product binding. Our methods have created high-activity enzymes with new functions (e.g. new sub-site activity). This process has also revealed a current limitation, however. Most of the enzymes with a new activity against a target substrate are not highly specific for only that substrate. Thus engineering high specificity is remains difficult. We have identified the structural basis for this substrate promiscuity with hydrophobic substrates but have not eliminated the problem. We have made recent progress, however, in engineering ionic interactions. The engineering challenge is that buried salt bridges are rare in nature and hard to engineer because the energy gained from the internal salt bridge must compensate for both desolvation of the charged groups and lost interactions with counter-ions in solution. We have overcome this by creating co-factor dependence of substrate binding. This is similar to our approach of engineering anion-triggered catalysis by manipulating active site amino acids. In this case, we have evolved a cation binding site in the S4 pocket which creates high specificity for a P4 glutamic acid. The principles involved in evolving this site are general to other enzymes.

3. Engineering activation, specificity and inhibition enables the creation of powerful enzymatic machines The ability to engineer a custom catalyst for any arbitrary chemical reaction obviously would be very useful. This remains a difficult challenge. We believe, however, that this vision of the problem is too narrow. If we view enzyme engineering as creating components that can be assemble into more complex machines, the task becomes tractable. A major, long-term benefit of this project will be the development of enzymatic components that can be assembled into multi-component enzymatic machines. The selection system we are using to evolve enzymatic function is itself a powerful analogue computer. The key component of this machine is a subtilisin with switchable activity. The selection machine parses random sequence space and "purifies" protease variants that not only specifically bind desired substrates but also efficiently perform the chemical steps required for peptide bond hydrolysis of the desired substrates. The "switchable" subtilisin is a general component that is

being applied to other enzymatic problems. Our quest to create absolute specificity has led to second key component of enzymatic machines. These are pairs of proteases with incongruent specificities. In an incongruent pair, one protease cuts substrate A but not B. The other cuts substrate B but not A. Such pairs are extremely useful in the construction of enzymatic logic gates. A third key component of enzymatic machines are tight inhibitors. Such inhibitors have been combined with sequence specific proteases to form complexes that can be either conditionally activate-able or self-amplifying. We are combining these three components to form sensors, switches, transducers and signal amplifiers. If one considers the construction of complex electronic devices from standard components, one can begin to see the potential of creating enzymatic machines from standard enzymatic components.

So what?

The proteases developed in this project will deactivate proteins toxins but will also be useful components of enzymatic machines. As the technology progresses, protease machines can be used for increasingly complex functions. This has implications for Bio-Defense because protease-based machines may eventually be used to both detect and destroy BWA toxins.

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